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Inhibition of herpes simplex virus infection by tannins and related compounds

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Summary

Several chemically defined plant extracts were investigated for their antiviral action on herpes simplex virus (HSV-1, HSV-2)-infected African green monkey kidney cells and human adenocarcinoma cells, using a plaque formation assay. Among them, the monomeric hydrolyzable tannins, oligomeric ellagitannins and condensed tannins, having galloyl groups or hexahydroxydiphenoyl groups, had the most potent anti-HSV activity. Their 50% effective doses (0.03–0.1 μg/ml) were by two-three orders of magnitude lower than their 50% cytotoxic doses (> 10 μg/ml). On the other hand, gallic acid, neutral polysaccharides, chemically modified (*N*,*N*-dimethylaminoethyl-, carboxymethyl-, and sulfated-) glucans, sialic acid-rich glycoproteins, and uronic acid-rich pine cone polysaccharide showed little or no activity. Using radiolabeled virus particles, we demonstrated that the anti-HSV effect of the tannins is due to inhibition of virus adsorption to the cells.

Tannin; Herpes simplex virus; Virus adsorption

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Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and -2) are responsible for a broad range of diseases in human, including gingivostomatitis, stomatitis, meningitis and venereally transmitted genital disease. After the primary infection, HSV tends to persist within the host. This is characterized as latent infection. The latent virus may be reactivated to cause recurrent herpetic infection (Whitley, 1985). Moreover, HSV has been reported to cause transformation of rodent cells (Hamper, 1981).

In contrast to several synthesized chemicals (Streissle et al., 1985), no detailed report of clinical utilization of the natural products is yet available. We recently found that various acidic antitumor substances of pine cones protect African green monkey kidney cells and human adenocarcinoma cells from the lethal effect of HSV infection, and that the spectral characteristics of these substances indicate the involvement of oxidizable phenolic groups and carboxylic acid groups in the molecules (Fukuchi et al., 1989). The oxidizable phenolic natural products may lead to tannins or lignins. Since the activities of tannins are often different from each other depending on their chemical structures, as exemplified by the antitumor activity specifically exhibited by some oligomeric hydrolyzable tannins with molecular weights over 1600 (Miyamoto et al., 1987), we investigated the antiviral activity of eleven chemically defined tannins and related polyphenols, and several natural and chemically modified antitumor polysaccharides.

Materials and Methods

Materials

The following 11 tannins and related polyphenols were isolated from the plants indicated in parentheses: oenothein B (*Oenothera erythrosepala* Borbas), coriariin A (*Coriaria japonica* A. Gray), rugosin D (*Rosa rugosa* Thunb.), cornusiin A (*Cornus officinalis* Sieb. et Zucc.), tellimagrandin I (*Casuarina stricta* Ait.), casuarictin (*Casuarina stricta* Ait.), geraniin (*Geranium thunbergii* Sieb. et Zucc.), licopyranocoumarin (*Glycyrrhiza* sp.), glycyrrhisoflavone (*Glycyrrhiza* sp.), 4,8-tetramer of epicatechin gallate (*Saxifraga stolonifera* Meerb.), and pentagalloylglucose (prepared from tannic acid JP (Japanese Pharmacopoeia) (Miyamoto et al., 1987; Hatano et al., 1986, 1988a, b, c) (Fig. 1). Paramylon, a polysaccharide with an unbranched β-1,3-D-glycopyranoside structure was extracted from *Euglena gracilis* (52000 kDa) (Miyatake et al., 1983), after which its chemically modified derivatives (*N,N*-dimethylaminoethylparamylon, carboxymethylparamylon and sodium paramylon sulfate) were synthesized (Unten et al., submitted for publication). PSK, a protein-bound polysaccharide prepared from the mycelium of CM-101 strain of *Coriolus versicolor* belonging to Basidiomycetes (Tsukagoshi et al., 1984), was provided by Kureha Chemical Company, Tokyo, Japan. Schizophyllan, a glucan from the culture filtrate of *Schizophyllum commun* (Komatsu et al., 1969), was

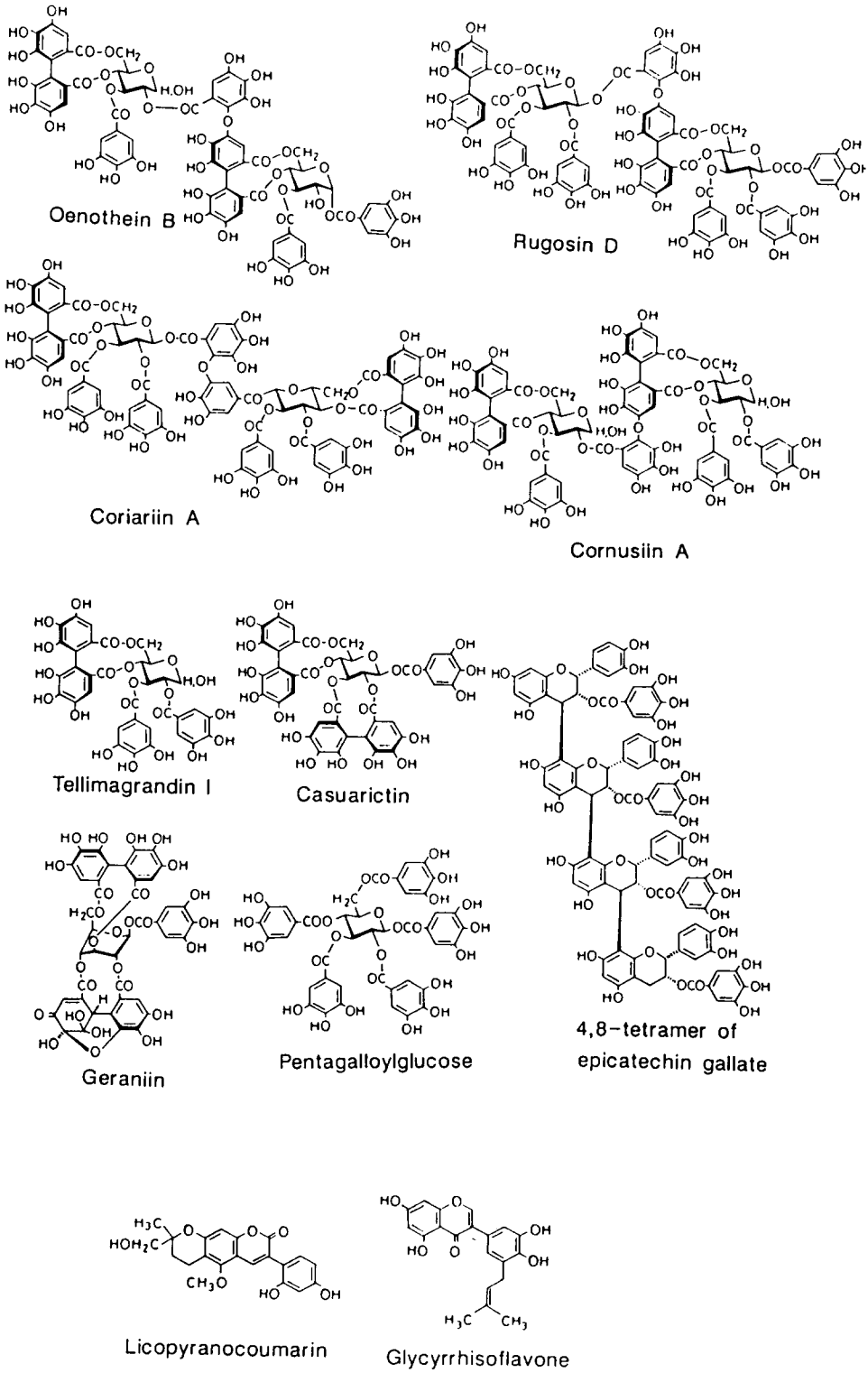

Fig. 1. Formulae of tannins and related compounds.

provided by Dr. N. Komatsu. CM-TAK, obtained by carboxymethylation of TAK, a glucan purified from *Alcaligenes faecalis* var. myxogenes, IFO 13140 (Sasaki et al., 1979), was provided by Takeda Chemical Industries, Osaka, Japan. Four sialic acid-rich glycoproteins were isolated from rainbow trout eggs: 200 kDa H-PSGP (sialic acid 60%) (Kitajima et al., 1986) and 9 kDa L-PSGP (sialic acid 60%) (Inoue et al., 1986); from *Plecoglossus altivelis* eggs, 9 kDa AL-SGP (sialic acid 20%) (Inoue et al., 1985); and from *Oryzias latipes* eggs, 7 kDa ML-SGP (sialic acid 23%) (Kitajima et al., 1989). The following reagents were used without further purification: tannic acid (Dainippon Pharmaceutical Company, Osaka, Japan), gallic acid (Wako Pure Chemical, Osaka, Japan), idoxuridine (IDU) (Kaken Pharmaceutical Company, Tokyo, Japan), and sodium dextran sulfate (Wako). Recombinant human tumor necrosis factor and interferon-γ were supplied by Dr. K. Takeda, Showa University. The various fractions of pine cone extract of *Pinus parviflora* (Frs. II, VI and VII) were prepared as reported previously (Sakagami et al., 1987).

Preparation of TX1, GT2 and GT3

Green tea (200 g) was washed successively with methanol and ethanol, and then extracted five times with hot water (70°C). After removal of insoluble material by centrifugation at 10000 × *g* for 30 min, the supernatant was concentrated by precipitation with 5 volumes of ethanol (TX1: Vmax (KBr) 3425, 1705, 1602, 1502, 1445, 1202, 1096, and 1040 cm^{-1}) (Tanuma et al., 1989). Residue which was not extracted with hot water was further extracted with 0.2 N NaOH for 6 h at room temperature. After centrifugation at 10000 × *g* for 30 min, the pH of the supernatant was adjusted to 5.0 with acetic acid. The precipitate was removed by centrifugation, and the supernatant was successively precipitated with 1 and 2 volumes of ethanol at 0–4°C (GT2 and GT3).

Viruses

The HSV-1 strain HF was supplied by Dr. Kanji Hirai, Tokai University. HSV-1 strain F and HSV-2 strain G were supplied by Dr. S. Arai, Okayama University. Virus stock was prepared by sonication of the HSV-1-infected CV-1 cells, and stored at −80°C until used (Hirai, 1970). The titer of virus, as determined by plaque formation assay, using CV-1 cells as target cells, was 1×10^7 plaque forming units (PFU) per ml.

Cells

African green monkey kidney cells (CV-1 (Jensen et al., 1964), VERO (Shimizu et al., 1967)) and human adenocarcinoma A-549 (Giard et al., 1973), obtained from the Japanese Cancer Research Resource Bank, were grown as monolayer cultures in Dulbecco's Minimum Essential Medium (DME) supplemented with 5% fetal calf serum (FCS).

Assay for plaque formation

To evaluate the direct inhibitory effect of test samples on HSV infection in the target cells, plaque assay was performed using agarose overlay with varying concentrations of the samples. Briefly, confluent monolayer cells – 1×10^6 cells in a 3.5 cm-diameter 6-well tissue culture plate (Corning) – were infected with the virus stock at 200–400 PFU per well for 1 h at 37°C in the absence (control) or presence of the test compounds. After allowing 1 h for virus adsorption, the infected cells were washed once with DME, and overlaid with 2 ml DME-2% FCS containing 0.5% sea plaque agarose (FMC Bioproducts, U.S.A.) without (control) or with test compounds. After incubation for 2 days at 37°C, the agarose overlay was removed, and the attached cells were stained with 0.2% crystal violet in 2% ethanol. The number of visible plaques was then counted under light microscopy. Portions of the cells were treated with test samples before or after virus infection, or at the time of virus infection, and incubated for 2 days at 37°C in the DME medium containing agarose as described above (for the experiment of Table 3). The anti-HSV activity of each test sample was determined as percent decrease in the number of plaques according to the following formula:

$$\% \text{ decrease of plaques} = \frac{\text{No. of plaques (control)} - \text{No. of plaques (tested)}}{\text{No. of plaques (control)}} \times 100.$$

Assay for cytotoxic activity

Monolayer CV-1 cells (4×10^4/l ml culture medium in a 24 well multidish (Nunc, InterMed)) were incubated with 0 (control), 1 10 or 30 μg/ml of the eleven samples of tannins and related compounds for the indicated periods. After detachment by trypsinization, the number of the viable cells was determined by trypan blue dye exclusion. The dose which decreased the number of viable cells by 50% (CD_{50}) was determined at day 3.

Assay for inhibition of virus adsorption

Confluent monolayer VERO cells (1×10^8 cells) were infected with HSV-1 (HF) at an MOI of 20. After 1 h, the infected cells were incubated for 24 h with 1 μCi/ml of [^{3}H]methyl-thymidine (New England Nuclear, Massachusetts, U.S.A.), and harvested by scraping with a rubber policeman. The cells were pelleted, suspended and disrupted by 10 strokes of a Dounce homogenizer (Type A pestle) to release the labeled virus particles. Cell debris and nuclei were removed by centrifugation at 3000 rpm for 5 min at 4°C, and the resulting supernatant (2 ml) was applied to 30 ml of 5–40% linear sucrose gradient in PBS, and centrifuged at 12500 rpm for 1 h at 4°C in a Beckman SW27 rotor. The band of radiolabeled virus was recovered, diluted with PBS, pelleted, and finally suspended in the growth me-

dium. To monitor virus adsorption, CV-1 cells (in 6-well plates) were incubated at 37°C for 1 h with virus suspension (60000 cpm) (20000 PFU/well) in the presence or absence of the test compounds, and the cell-bound radioactivity was then determined in a liquid scintillation counter after vigorous washing with DME (3 times).

Results

Inhibitory effect of tannins on HSV-1 plaque formation

Inhibition of HSV infection was determined by plaque assay. Cells were inoculated with HSV-1 (200–400 PFU/well), usually to form 200–400 plaques per well (3.5 cm diameter) 2 days after inoculation. We previously found that two distinct antitumor substances (10 kDa Fr. VI and 70–200 kDa Fr. VII) isolated from aq. NaOH extract of pine cone of *Pinus parviflora* Sieb. et Zucc. (Sakagami et al., 1987) significantly inhibited herpes simplex virus type 1 and 2 (HSV-1 and -2) plaque formation in the CV-1 cells (Fukuchi et al., 1989), the ED_{50} (dose that inhibited plaque formation by 50%) being 0.31 and 0.46 μg/ml, respectively (Table 1).

We found that TX1, GT2 and GT3, isolated from green tea by a similar procedure, had anti-HSV activity almost comparable with that of Fractions VI and VII. From the dose response curve, ED_{50} of TX1 was calculated to be 0.1 μg/ml (Fig. 2). Since the IR absorption curve of TX1 was found to be very similar to that of the commercially available tannic acid (see Fig. 1 in Tanuma et al., 1989), we next investigated the anti-HSV activity of a variety of chemically defined tannins (formulae as depicted in Fig. 1). As expected, the anti-HSV activity of tannic acid was very potent (ED_{50}: 0.034 μg/ml). Oenothein B (Fig. 2), coriariin A, rugosin D, cornusiin A, tellimagrandin I, casuarictin and 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose, a methanolysis product of tannic acid (Miyamoto et al., 1987), had activity almost comparable to that of tannic acid (ED_{50}: 0.034–0.047 μg/ml) (Table 1). The activity of geraniin and the 4,8-tetramer of epicatechin gallate was slightly lower (ED_{50}: 0.093–0.14 μg/ml). Polyphenols with low molecular weights, licopyranocoumarin, glycyrrhisoflavone and gallic acid – a component of tannin (Knudson, 1913) – had little or no activity (ED_{50} >10 μg/ml).

It should be noted that a commercially available lignin, another polyphenolic substance known as a main constituent of wood, showed significant anti-HSV activity at a slightly higher concentration (ED_{50}: 1.4 μg/ml).

On the other hand, the following substances showed no significant anti-HSV activity at concentrations up to 10 μg/ml: three neutral antitumor polysaccharides (Paramylon, PSK and Schizophyllan), chemically modified glucans (*N,N*-dimethylaminoethyl paramylon, sodium carboxymethyl paramylon, sodium paramylon sulfate and carboxymethyl TAK), four sialic acid-rich glycoproteins (H-PSGP, L-PSGP, AL-SGP and ML-SGP), Fraction II (pine cone water-extractable polysaccharide containing large amounts of uronic acid), and two cytokines [tumor

TABLE 1

Inhibition of HSV-1 (strain HF) plaque formation by tannins

	Anti-HSV activity[a] (ED_{50}: μg/ml)	Cytotoxic activity[b] (CD_{50}: μg/ml)
Plant extracts		
Fraction VI (pine cone)	0.31	
Fraction VII (pine cone)	0.46	
TX1 (green tea)	0.10	
GT2 (green tea)	4.2	
GT3 (green tea)	0.52	
Tannins and related compounds		
Tannic acid (commercial)	0.034	18
Oenothein B	0.036	>30
Coriariin A	0.038	>30
Rugosin D	0.034	>30
Cornusiin A	0.039	>30
Tellimagrandin I	0.036	>30
Casuarictin	0.044	>30
Penta-*O*-galloyl-β-D-glucose	0.047	7
Geraniin	0.093	>30
4,8-Tetramer of epicatechin gallate	0.14	>30
Licopyranocoumarin	>10	23
Glycyrrhisoflavone	>10	15
Gallic acid	>10	
Others		
Lignin (commercial)	1.4	
Fraction II (pine cone)	>10	
IDU	16	
Sodium dextran sulfate	3.2	

[a]CV-1 cells were infected with HSV-1 strain HF in the presence of various concentrations of the test samples. The 50% effective doses of tannin derivatives were then determined from titration curves like those presented in Fig. 2.

[b]CV-1 cells were incubated for 3 days with various concentrations of tannin derivatives and their 50% cytotoxic doses were then determined from growth curves like those presented in Fig. 3.

Each value represents mean for two separate determinations.

necrosis factor (10 ng/ml) and interferon-γ (10 units/ml)] (data not shown). However, IDU, a clinically utilized anti-HSV agent, and sodium dextran sulfate, a potent inhibitor of human immunodeficiency virus infection and replication (Nakashima et al., 1987), completely inhibited HSV-1 plaque formation at higher concentrations (ED_{50} 16 and 3.2 μg/ml, respectively) (Table 1). The results obtained for dextran sulfate are in agreement with those of Baba et al. (1988).

Table 2 shows that tannic acid, at concentrations of 0.034–0.15 μg/ml, significantly inhibited plaque formation by the two different HSV-1 strains (HF and F) and the HSV-2 strain G in monkey kidney (CV-1 and VERO) cells and human adenocarcinoma (A-549) cells.

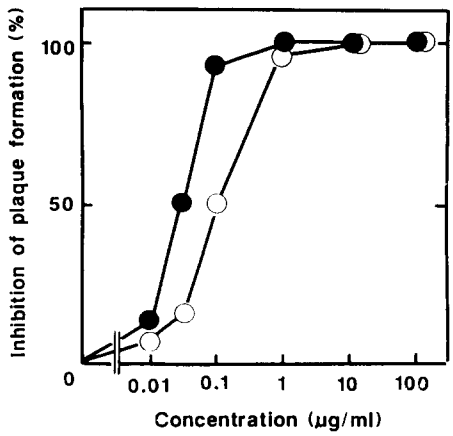

Fig. 2. Inhibitory effects of TX1 and oenothein B on HSV plaque formation in CV-1 cells. CV-1 cells were infected with HSV-1 (strain HF) and treated with the indicated concentrations of TX1 (○) or oenothein B (●).

Effect of tannins and related compounds on the growth of CV-1 cells

We investigated the effects of various tannin derivatives on the growth of CV-1 cells. As shown in Fig. 3, neither the growth rate nor the saturation density of CV-1 cells was significantly affected at a concentration of 10 μg/ml of oenothein B, one of the most potent anti-HSV agents among the tannins, although the plating efficiency of CV-1 cells declined slightly at 30 μg/ml. Similar cytotoxic effects of other active tannins, such as coriariin A, rugosin D, cornusiin A, tellimagrandin I, casuarictin, 4,8-tetramer of epicatechin gallate, geraniin were observed only at concentrations higher than 30 μg/ml (Table 1). It should be noted that the inactive phenolics, such as licopyranocoumarin and glycyrrhisoflavone, were relatively cytotoxic, their 50% cytotoxic doses being 23 and 15 μg/ml, respectively (Table 1).

TABLE 2

Inhibitory effect of tannic acid on plaque formation by various HSV strains infected in three different target cells

Target cells	strain	ED_{50} (μg/ml)
CV-1	HSV-1 (HF)	0.034
CV-1	HSV-1 (F)	0.15
CV-1	HSV-2 (G)	0.15
Vero	HSV-1 (HF)	0.086
Vero	HSV-1 (F)	0.043
Vero	HSV-2 (G)	0.041
A-549	HSV-1 (F)	0.061

The cells were infected with various HSV strains in the presence of various concentrations of tannic acid, and the ED_{50} value was determined as described in Materials and Methods. Each value represents mean for two separate determinations.

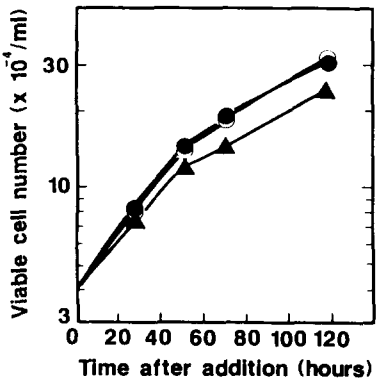

Fig. 3. Effect of oenothein B on CV-1 cell growth. CV-1 cells were incubated with oenothein B at 0 (○), 10 (●) or 30 μg/ml (▲) for the indicated times and the number of viable cells was determined by trypsin blue dye exclusion.

Inhibition of virus adsorption by tannin

To determine the stage of HSV-1 infection that was inhibited by tannins, cells were treated with tannic acid at various times before and after HSV-1 infection. Table 3 shows that an inhibitory effect was observed only when tannic acid was added during virus adsorption.

When CV-1 cells were exposed to radiolabeled HSV particles in the absence or presence of tannic acid and the cell-bound radioactivity was then determined (Table 4), tannic acid (at 10 μg/ml) appeared to inhibit cell-binding of the radiolabeled virus particles, even when the cells were infected with virus at 20000 PFU/well. However, we cannot at present exclude the possibility that tannins might directly inactivate the virus, resulting in a reduced ability of the virus particles to attach to the cell membrane.

We then investigated the effect of tannic acid on HSV penetration. Cells were

TABLE 3

Dependence of anti-HSV activity of tannic acid on treatment schedule

Tannic acid (μg/ml) present			No. of plaques	Percent inhibition
Before adsorption	During adsorption	After adsorption		
0	0	0	255	–
1	0	0	250	0
10	0	0	267	0
0	1	0	0	100
0	10	0	0	100
0	0	1	270	0
0	0	10	248	0

CV-1 cells were infected with HSV-1 strain HF. Each value represents mean for two separate determinations.

TABLE 4

Inhibitory effect of tannic acid on adsorption of radiolabeled virus to the cells

Tannic acid (μg/ml)	Cell-bound radioactivity (cpm/well)
0	5980 ± 285
10	1460 ± 203

CV-1 cells were incubated for 1 h at 37°C with radiolabeled virus equivalent to 60000 cpm (20000 PFU/well) in the absence or presence of tannic acid, and the cell-bound radioactivity was then determined as described in Materials and Methods. Each value represents mean ± SE for 3 samples.

exposed for 1–2 h to virus at 4°C, a condition that allows virus adsorption but not virus penetration (Highlander et al., 1987). The cells were then treated with tannic acid and the temperature was raised to 37°C to allow virus penetration. Under these conditions tannic acid (1 μg/ml) did not inhibit plaque formation. Taken together these data demonstrate that tannic acid inhibits virus adsorption to the target cells but does not inhibit virus penetration.

Discussion

We have demonstrated that chemically defined tannins significantly inhibit plaque formation of various HSV strains in both monkey and human cells. Their 50% effective doses are 2 to 3 orders of magnitude lower than their respective 50% cytotoxic doses (Table 1). Our results confirm previous observations on the anti-HSV activity of tannins (Takechi et al., 1985). Apparently, the presence of polyphenol groups is an important determinant in the anti-HSV activity of the tannins.

It is interesting that the anti-HSV activity of the monomeric hydrolyzable tannins (> 500 kDa) [tellimagrandin I (787 kDa), casuarictin (937 kDa), geraniin (953 kDa)], oligomeric ellagitannins [coriariin A (1875 kDa), oenothein B (1571 kDa), rugosin D (1875 kDa), cornusiin A (1571 kDa)] and condensed tannin [4,8-tetramer of epicatechin gallate (1763 kDa)], all of which contain three hydroxyl groups in the benzene ring, was greater than that of licopyranocoumarin (384 kDa) and glycyrrhisoflavone (368 kDa), which contain only two hydroxyl groups in the benzene ring. This suggests that the number of hydroxyl groups in the benzene ring is important in determining the extent of anti-HSV activity. The low virus-inhibitory activity of lignin can be attributed to the absence of a trihydroxylated benzene ring (Harborne 1973). We also found that gallic acid (170 kDa), a component of various tannins, had no anti-HSV activity, although it has three hydroxyl groups. This suggests that the anti-HSV activity of the tannins not only depends on the number of polyphenolic groups but also on the molecular weight of the molecule. The capacity to bind to proteins (Okuda et al., 1985) and antitumor activity (Miyamoto et al., 1987) of tannins also depend on their molecular weight. It is noteworthy that dimeric ellagitannins with molecular weights higher than 1600, which have strong anti-HIV activity (Asanaka et al., 1988), also demonstrate potent anti-HSV activity (Table 1). It is also noteworthy that the eleven chemically

defined tannins used here (Table 1) were not markedly cytotoxic, which contracts with the previously reported tannins (Takechi et al., 1985). Although several of the high-molecular-weight tannins, which show potent anti-HSV activity, contain sugar, the presence or absence of the sugar does not correlate with anti-HSV activity. In fact, the 4,8-tetramer of epicatechin gallate, which lacks the sugar moiety, is quite active against HSV (Table 1).

We have previously reported that tannins and related compounds inhibited tumor promotion in a two-stage carcinogenesis experiment (Yoshizawa et al., 1987), and are also endowed with anti-HIV activity (Hatano et al., 1988a). Inhibitory effects of tannins on the reverse transcriptase activity associated with RNA tumor viruses (Kakiuchi et al., 1985) and poly- (ADP-ribose-) glycohydrolase activity of human placenta (Tanuma et al., 1989) have also been reported. The present study adds further credence to the antiviral potential of tannins. The structure-function relationship that underlies the anti-HSV activity of tannins remains to be elucidated.

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